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Effects of Cinnabar on Pyrite Oxidation by *Thiobacillus ferrooxidans* and Cinnabar Mobilization by a Mercury-Resistant Strain

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The effect of cinnabar on pyrite oxidation by mercury-sensitive and mercury-resistant strains of *Thiobacillus ferrooxidans* was investigated by using percolation columns. Mercury-resistant strains oxidized pyrite in pyrite-cinnabar mixtures (1 and 10%, wt/wt), whereas a mercury-sensitive strain did not. Elemental mercury was produced by the mercury-resistant strains growing in the pyrite-cinnabar mixtures in percolation columns and in flasks containing cinnabar only. Manometric experiments showed that cinnabar had little effect on oxygen uptake of mercury-sensitive or mercury-resistant cells growing on ferrous sulfate, pyrite, or pyrite-ferrous sulfate mixtures. In addition, shake flask leaching experiments showed that cinnabar had little effect on pyrite oxidation at 1% (wt/wt) but inhibited growth of mercury-sensitive and mercury-resistant strains at 10%. Mercury-resistant strains were unable to grow on cinnabar as an energy source.

Cinnabar (HgS) is the major mercury-bearing mineral in nature (1, 11), and its mobilization is an important step in the natural mercury cycle. However, very little is known about cinnabar mobilization by bacteria. Small amounts of methylmercury species can be formed from cinnabar in aerobic sediments (12) and in aqueous systems through photochemical reactions with amino acids (14).

It has been assumed (8) that metal sulfide-oxidizing bacteria such as the thiobacilli slowly oxidize HgS, but this has received almost no experimental attention (26). Torma and Sakaguchi (27) found that the solubility of metal sulfides was related to their ability to be oxidized by the commercially important metal sulfide ore-leaching bacterium *Thiobacillus ferrooxidans*. Consequently, HgS, with its extremely low solubility in water, is expected to undergo only very slow attack by this organism. Accordingly, Silver and Torma (22), in a study of microbial oxidation of a variety of metal sulfide minerals, observed no oxygen uptake by *T. ferrooxidans* cells incubated with HgS as the sole substrate. However, some Hg was solubilized (<4 ppm [4 μg/g]) from HgS by *T. ferrooxidans* grown on metal sulfide minerals, with a larger amount of Hg solubilized (64 ppm) with $FeSO_4$-grown cells (22).

Mercury is highly toxic to *T. ferrooxidans* (15, 18), and its presence in ore deposits may inhibit the important activity of thiobacilli in commercial leaching operations (7). However, mercury-resistant strains of *T. ferrooxidans* which volatilize mercury have recently been reported (6, 18, 19, 21).

The purpose of this investigation was to determine whether mercury-resistant *T. ferrooxidans* may mobilize Hg from cinnabar and to determine the effects of cinnabar on pyrite and iron oxidation by mercury-sensitive and mercury-resistant strains of *T. ferrooxidans*.

MATERIALS AND METHODS

Organisms and culture conditions. Isolates of *T. ferrooxidans* were obtained from sediments of pyrite mines at Campiano, Tuscany, Italy (2). Pure cultures (13) were obtained by single-colony isolations on ISP agar (17). ISP medium was prepared by combining three separately sterilized solutions: solution A (300 ml), containing 33.4 g of $FeSO_4 \cdot 7H_2O$ per liter of deionized water, adjusted to pH 2.5 with 6 M H_2SO_4 and filter sterilized; solution B (550 ml), containing (per liter) 6.0 g of $(NH_4)_2SO_4$, 0.2 g of KCl, 1.0 g of $MgSO_4 \cdot 7H_2O$, and 0.02 g of $Ca(NO_3)_2$, adjusted to pH 3.0 and autoclaved at 121°C for 15 min; and solution C, containing 7.0 g of purified agar L28 (Oxoid USA, Inc., Columbia, Md.) in 150 ml of deionized water and autoclaved at 121°C for 15 min. *T. ferrooxidans* 13661 was kindly provided by R. M. Kelly, Johns Hopkins University, and strain BA-4 was isolated from a coal mine settling pond in Wyoming as described previously (18). Cultures of *T. ferrooxidans* were routinely grown in 9K liquid medium (23) in shake flasks (100 ml of medium in 250-ml Erlenmeyer flasks) on a rotary shaker (29°C, 250 rpm) or in 1.2-liter bottles containing 1.0 liter of 9K medium and a glass tube packed with sterile glass wool through which compressed air was passed to vigorously aerate the solution. The 9K medium was prepared by combining two separately autoclaved (121°C, 15 min) solutions: basal salts, containing (in 700 ml of deionized water) 3.0 g of $(NH_4)_2SO_4$, 0.10 g of KCl, 0.50 g of K_2HPO_4, 0.50 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $Ca(NO_3)_2$, and 1.0 ml of 10 N H_2SO_4; and the energy source, 300 ml of a 14.74% (wt/vol) solution of $FeSO_4 \cdot 7H_2O$ in deionized water.

Organisms were also grown with pyrite >85% FeS_2, −80 mesh, <177 μm; Matheson, Coleman and Bell, Norwood, Ohio at a concentration of 10% (wt/vol) in 9K salts (pH 2.5) containing 0.1% Fe (as $FeSO_4 \cdot 7H_20$). The pyrite was analyzed by scanning electron microscopy with energy dispersive X-ray microanalysis. Some copper (<1.0% by weight) was present in the pyrite along wth Al, Si, and K. No other heavy elements were detected. Cinnabar crystals were obtained from cinnabar mines at Abbadia San Salvatore, Tuscany, and ground to −200 mesh (<74 μm) with a mortar and pestle. The powdered material was >95% HgS with no detectable pyrite, marcasite, or elemental sulfur as shown by X-ray diffraction analysis. Minerals were autoclaved at 121°C for 15 min.

MICs of mercury were determined by transferring 0.1 ml of log-phase cells in 9K medium into 10 ml of fresh 9K

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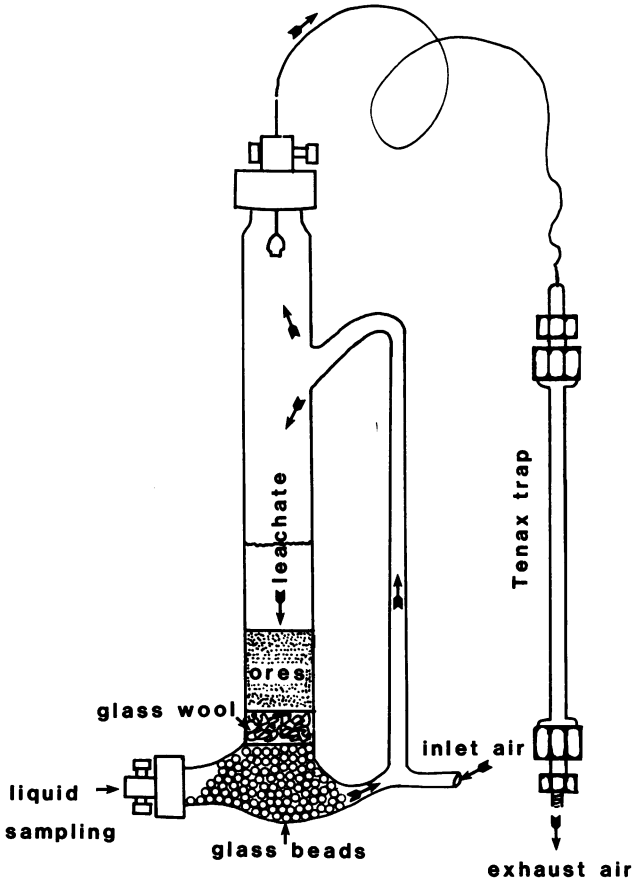

FIG. 1. Airlift leaching column used for pyrite-cinnabar oxidation studies. Ores were supported on glass beads and glass wool. Air was exhausted through the column cap and through a trap containing Tenax GC resin to trap mercury compounds. After sampling, the trap was removed and attached to a purge and trap sampler, and mercury compounds on the trap were desorbed into a GC-AA system for Hg speciation.

medium containing various concentrations of $HgCl_2$. After 7 h, ferrous iron (substrate) concentrations were determined by titration with permanganate (24). Mercury-resistant cells were induced by preincubation in 9K medium containing $HgCl_2$ (0.3 μg of Hg per ml).

Leaching studies. Pyrite oxidation studies were performed with glass airlift percolation columns (Fig. 1). The columns were loaded with 30 g of pyrite mixed with various amounts of cinnabar onto a bed of glass wool and glass beads. After autoclaving (121°C, 15 min), 30 ml of sterile 9K salts (pH 2.5) plus 0.1% Fe (as $FeSO_4 \cdot 7H_2O$) was added to the columns, and they were inoculated with approximately 10^9 cells of *T. ferrooxidans*. The inoculum was prepared by centrifugation of 50 ml of late-log-phase cells in 9K medium (6,000 × *g*, 10 min), suspension of the cell pellet in 9K salts, and a second centrifugation and suspension in a small amount of 9K salts. Compressed air was passed through two in-line charcoal (sugar-charcoal) traps into the percolation columns. At certain intervals, the exhaust air was passed through the top cap (Mininert cap; Supelco, Inc., Bellefonte, Pa.) of the columns via a syringe needle and small-bore (1.0-mm inner diameter) Teflon tubing and through stainless steel tubes packed with 60-80 mesh Tenax GC resin (Alltech Associates, Deerfield, Ill.) to trap volatile Hg compounds for subsequent chemical speciation of mercury (2) (see below).

Leaching experiments were also conducted by shake flask techniques, with 250-ml Erlenmeyer flasks filled with 30 g of pyrite, 30 ml of 9K salts, 0.1% Fe, and various amounts of cinnabar.

Chemical determinations. Total acidity of pyrite-leaching solutions was determined by titration of hot, freshly boiled dilutions (1:50 in deionized water) of leaching solutions to the phenolphthalein endpoint with 0.05 N NaOH. Ferrous iron in solution was determined by titration with permanganate (24). Mercury in solution was determined by the method of additions by a cold-vapor procedure (28) or by using a purge-and-trap sampler with a Tenax trap column (model 7675 A; Hewlett Packard, Avondale, Pa.). Mercury species were desorbed from the sampler by heating and carried by N_2 through heated transfer lines to a gas chromatograph-atomic absorption (GC-AA) detection system (2, 5, 18) for chemical speciation of the mercury. The detection limit (20) for total mercury in solution varied depending on sample size (1 to 20 ml) and ranged from 1.4 ng/ml for leaching-column samples to 0.07 ng/ml for shake flask samples.

Manometric studies. Reactions were run in dual-side-arm respirometer vessels in a differential respirometer (Gilson Medical Electronics, Middleton, Wis.). Each vessel contained 2.7 ml of 9K salts and 0.3 ml of deionized water or a 5% (wt/vol) solution of $FeSO_4 \cdot 7H_2O$. The center wells contained 0.2 ml of 20% KOH and filter paper wicks. Solid substrates (pyrite [3.0 g] or cinnabar [0.1 g]) were added to solutions, and cells (0.3 ml in 9K salts) were tipped into the reaction mixture after 30 min of preincubation at 28°C and shaking at 140 strokes per min. Cell suspensions were about 2×10^8 to 6×10^8 cells per ml (final concentration) in reaction vessels. Uptake rates were calculated from linear portions of O_2 uptake curves.

RESULTS

We isolated three rod-shaped, iron-oxidizing, acidophilic bacteria, identified as *T. ferrooxidans*, from metal sulfide deposits in Italy. The mercury resistance ($HgCl_2$) of these strains was compared with that of *T. ferrooxidans* 13661, not previously exposed to Hg, and strain BA-4, which is Hg resistant by virtue of mercuric reductase (19). After 1 week of incubation in 9K medium, all strains completely oxidized the ferrous iron substrate in the presence of 0.1 μg of Hg (as $HgCl_2$) per ml, but only strain BA-4 and one strain from Italy (designated SW9K1) oxidized iron in the presence of 0.5 μg of Hg per ml. Strains 13661, BA-4, and SW9K1 were chosen for further investigation.

The MICs of $HgCl_2$ for iron oxidiation showed 50% inhibition of strain 13661 at 0.2 μg of Hg per ml, with strains SW9K1 and BA-4 showing about 10-fold less sensitivity to Hg (Fig. 2). Analysis of headspace gases above the cultures by GC-AA showed that strains SW9K1 and BA-4 produced elemental Hg but strain 13661 did not (data not shown). After the introduction of uninduced cells of strain SW9K1 into 9K medium containing Hg (1 μg/ml), immediate production of Hg^0 was noted, suggesting that the mercury-reducing system in this strain is constitutive, as it is in BA-4 (19).

Leaching experiments and Hg volatilization. All cultures of *T. ferrooxidans* oxidized pyrite in shake flasks and leaching columns, as shown by an increase in the acidity of the solutions in the inoculated columns with time. In repeated experiments, strain SW9K1 also oxidized pyrite in columns containing 1 or 10% (wt/wt) cinnabar mixed with the pyrite,

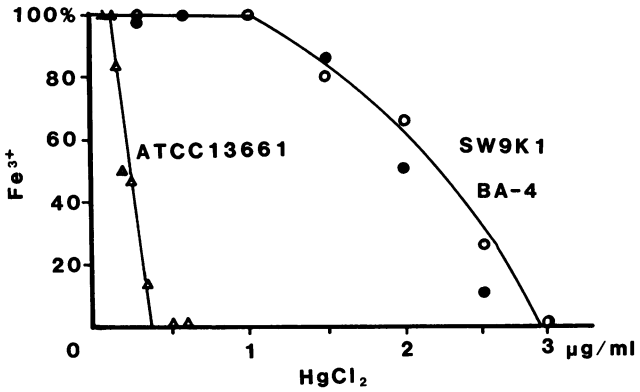

FIG. 2. MIC of $HgCl_2$ for iron oxidation by *T. ferrooxidans* strains. Induced log-phase cells were diluted 1:100 into fresh 9K medium containing different concentrations of $HgCl_2$, and the amount of iron oxidized after 7 h was measured.

but mercury-sensitive strain 13661 was completely inhibited in pyrite oxidation in the presence of 1 or 10% cinnabar (Fig. 3). During an interval in these experiments (days 23 to 30), gases from the leaching columns were passed through Tenax traps. Analysis of the traps by GC-AA for Hg compounds showed that the column inoculated with strain SW9K1 produced a total of 280 ng of Hg^0, whereas only 6 ng of Hg^0 was detected in the column inoculated with strain 13661 and 1 ng in the uninoculated column. The total volume of air passed through the traps during the 7-day period was 1,344 liters from the two inoculated columns and 2,940 liters from the uninoculated column. No soluble mercury was detected in leachates from either inoculated or uninoculated columns at either 1 or 10% cinnabar in pyrite. To confirm the production of Hg^0 from cinnabar, we inoculated strain SW9K1 into 50 ml of 9K salts (minus iron) plus 1.5 g of HgS (final cell concentration, 10^8/ml) and incubated the cells at 29°C on a rotary shaker (250 rpm). After 2 days, the culture

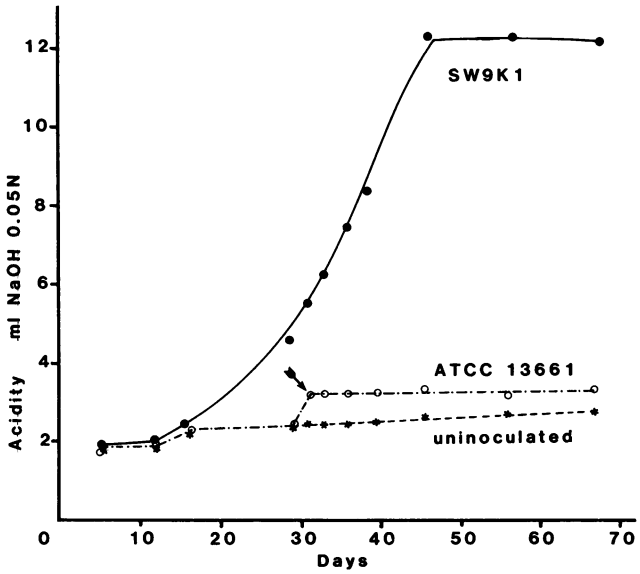

FIG. 3. Oxidation of pyrite in leaching columns in the presence of cinnabar. At time zero, 10^9 cells were added to leaching columns containing 30 g of pyrite and 3 g of HgS. At the time indicated (arrow), strain 13661 was reinoculated. Incubation was at 22°C.

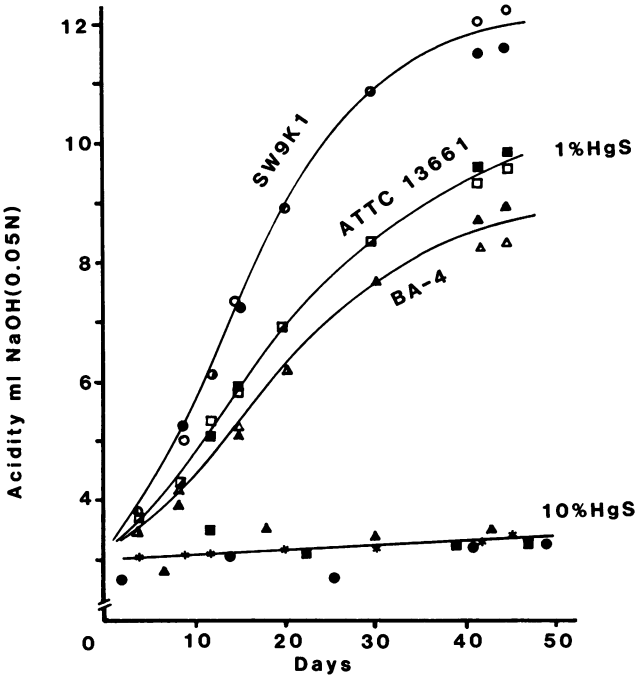

FIG. 4. Oxidation of pyrite alone (open symbols) and in the presence of cinnabar (solid symbols) by strains SW9K1 (circles), 13661 (squares), and BA-4 (triangles). Shake flasks were inoculated with 10^9 cells, and acidity measurements were made over time. *, Uninoculated control.

was vigorously bubbled with compressed air to remove any Hg^0 and then sealed for 15.5 h, after which time the headspace gas was analyzed by AA for Hg. With strain SW9K1, 39 ng of Hg^0 was detected in the headspace (150 ml total), whereas no Hg^0 (<1.4 ng) was detected above strain 13661 and a trace of Hg^0 was detected (about 1.3 ng) above an uninoculated solution. Soluble Hg was 6.8 ng/ml with strain SW9K1 and 2.1 ng/ml with strain 13661.

In shake flask experiments, complete inhibition of pyrite oxidation by strains BA-4, SW9K1, and 13661 occurred in flasks containing 10% cinnabar in pyrite. However, at 1% cinnabar in pyrite, there was pyrite oxidation by all three strains (Fig. 4). At the end of the experiments, there was no detectable soluble Hg in the 1% cinnabar flasks (<0.07 ng/ml) and 0.1 to 0.3 ng/ml in the 10% cinnabar flasks.

It was not possible to maintain cultures of any of the strains of *T. ferrooxidans* on cinnabar as the sole energy source in 9K salts (minus iron) at pH 2.5

Manometric studies. Cells of all three strains of *T. ferrooxidans* oxidized Fe^{2+} with O_2 uptake rates of 1,841 to 2,412 µl of O_2 per 10^9 cells per h (Table 1). Oxygen uptake by strain SW9K1 was inhibited about 50% in the presence of pyrite. Others have reported that pyrite and other fine particles can inhibit iron oxidation by *T. ferrooxidans* (10, 25). Cinnabar had little effect on O_2 uptake by strain SW9K1 or 13661. Very slight O_2 uptake by strain SW9K1 on cinnabar was noted, but no O_2 uptake by the other two strains was observed in the presence of cinnabar.

DISCUSSION

Little is known about the mobilization of cinnabar in nature, but it is often assumed that bacteria, especially metal

TABLE 1. Oxygen uptake by *T. ferrooxidans* strains

Strain	Mean O_2 uptake (μl/h per 10^9 cells) ± SD with substrate[a]:							9K salts
	Fe^{2+}	Fe^{2+} + pyrite	Fe^{2+} + cinnabar	Fe^{2+} + pyrite + cinnabar	Pyrite	Pyrite + cinnabar	Cinnabar	
SW9K1	1,841 ± 242	809 ± 14	1,750 ± 23	788 ± 296	376 ± 18	365 ± 17	14 ± 1	0
BA-4	2,141 ± 34	NT[b]	NT	NT	NT	NT	0	0
13661	2,412 ± 201	2,314 ± 169	2,297 ± 194	1,960 ± 810	424 ± 138	384 ± 124	0	0

[a] Vials contained 2.7 ml (3 ml in vials with cinnabar as the sole substrate) of 9K salts and 0.3 ml of 5% $FeSO_4 \cdot 7H_2O$, 3 g of pyrite, or 0.1 g of cinnabar as indicated.
[b] NT, Not tested.

sulfide-oxidizing thiobacilli, are involved in its transformation. *T. ferrooxidans* is known to oxidize a wide variety of metal sulfides, obtaining energy from the sulfide portion of the molecule (7), and thus could be a likely candidate for cinnabar oxidation. However, mercury is toxic to many strains of *T. ferrooxidans* at low levels (15, 18). This suggests that *T. ferrooxidans* and other metal sulfide ore-leaching bacteria might be inhibited in ore deposits containing mercury-bearing minerals (7). The only study, to our knowledge, that included cinnabar in studies on microbial metal sulfide oxidation found that cinnabar was not oxidized by *T. ferrooxidans* and that rather low levels of Hg were solubilized by the organism (22).

Recent reports (6, 18, 19) of mercury-resistant (volatilizing) *T. ferrooxidans* suggest that these strains might be more likely to mobilize cinnabar. We tested mercury-sensitive and mercury-resistant strains of *T. ferrooxidans* for pyrite oxidation (an important substrate for the organism in nature) in the presence of cinnabar and for cinnabar mobilization.

Leaching-column results, which may more closely resemble natural ore-leaching environments than shake flasks, showed that pyrite oxidation by non-Hg-resistant strain 13661 was inhibited by the presence of cinnabar, despite the fact that no soluble Hg (<1.4 ng/ml) was detected in column leachates. Mercury-resistant (volatilizing) strains SW9K1 and BA-4 oxidized pyrite in leaching columns at concentrations as high as 10% cinnabar in pyrite and produced Hg^0 in the process. However, HgS apparently cannot serve as a growth substrate for either mercury-resistant or mercury-sensitive strains. Very little or no O_2 uptake was noted in manometric experiments, confirming the observation that HgS is not an energy source for growth, even in Hg-resistant bacteria.

The results with cinnabar-pyrite mixtures in shake flasks are more difficult to interpret. However, part of the explanation may lie in the inherent dissimilarity of shake flasks and column-leaching techniques for evaluating microbial mineral sulfide (i.e., pyrite) oxidation. *T. ferrooxidans* cells adhere to pyrite surfaces during pyrite oxidation (4; T. Y. Yeh, J. R. Godshalk, G. J. Olson, and R. M. Kelly, Biotechnol. Bioeng., in press). However, shaking or agitation results in many fewer adherent cells (T. Y. Yeh, R. M. Kelly, and G. J. Olson, unpublished observations). Since aqueous solutions of Hg^{2+} and Hg^0 are adsorbed to pyrite surfaces (9), perhaps attached Hg-sensitive cells are inhibited by Hg on pyrite surfaces (leaching columns) in pyrite–1% cinnabar mixtures, whereas cells in free solution (more common shake flasks) that oxidize dissolved ferrous ions are not. This might explain the inhibition of *T. ferrooxidans* in leaching columns but not in shake flasks containing 1% cinnabar–pyrite mixtures. At 10% cinnabar–pyrite, all strains were inhibited in shake flasks.

Other growth effects are associated with particles in shake flask cultivation of *T. ferrooxidans*. For example, Soljanto et al. (25) found that finely ground particles, including pyrite and presumably inert glass beads, inhibited iron oxidation by *T. ferrooxidans*, the effects varying with particle concentration and size. The effect was less pronounced in static cultures. Dispirito et al. (10) extended this work and found that pH had an effect on particle inhibition. They suggested that the activity of *T. ferrooxidans* at the solid-liquid interface may be different from that in solution. The mechanisms of particle inhibition in shake flasks remains uncertain, and more work is required to understand metal sulfide oxidation mechanisms in shake flask versus column bioleaching environments.

Although a relatively small percentage of mercury was mobilized from cinnabar by *T. ferrooxidans*, this represents a potential pathway of cinnabar mobilization in nature.

The results of the leaching-column experiments suggest that non-mercury-resistant *T. ferrooxidans* could be inhibited in ores containing cinnabar. This may not affect commercial ore-leaching operations, since mercury-resistant strains are often selected in mercury-impacted environments (3, 16). However, potential future technologies with controlled leaching bioreactors and starter cultures should consider using mercury-resistant strains when the mercury content of substrates is elevated.

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